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Comparison on in vitro fertilized bovine embryos cultured in KSOM or SOF and cryopreserved by slow freezing or vitrification

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Abstract

The objectives of this study were to identify an improved in vitro cell-free embryo culture system and to compare post-warming development of in vitro produced (IVP) bovine embryos following vitrification versus slow freezing. In Experiment 1, non-selected presumptive zygotes were randomly allocated to four medium treatments without co-culture: (1) SOF + 5% FCS for 9 days; (2) KSOM + 0.1% BSA for 4 days and then KSOM + 1% BSA to Day 9; (3) SOF + 5% FCS for 4 days and then KSOM + 1% BSA to Day 9; and (4) KSOM + 0.1% BSA for 4 days and then SOF + 5% FCS to Day 9. Treatment 4 (sequential KSOM–SOF culture system) improved ($P > 0.05$) morulae (47%), early blastocysts (26%), Day-7 blastocysts (36%), cell numbers, as well as total hatching rate (79%) compared to KSOM alone (Treatment 2). Embryos cultured in KSOM + BSA alone developed slowly and most of them hatched late on Day 9, compared to other treatments. In Experiment 2, the sequential KSOM–SOF culture system was used and Day-7 blastocysts were subjected to following cryopreservation comparison: (1) vitrification (VS3a, 6.5 M glycerol); or (2) slow freezing (1.36 M glycerol). Warmed embryos were cultured in SOF with 7.5% FCS. Higher embryo development and hatching rates ($P < 0.05$) were obtained by vitrification at 6 h (71%), 24 h (64%), and 48 h (60%) post-warming compared to slow freezing (48, 40, and 31%, respectively). Following transfer of vitrified embryos to synchronized recipients, a 30% pregnancy rate was obtained. In conclusion, replacing KSOM with SOF after 4 days of culture produced better quality

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blastocysts. Vitrification using VS3a may be used more effectively to cryopreserve in vitro produced embryos than the conventional slow freezing method.

1. Introduction

The pioneering work of Tervit et al. [1] and Lawitts and Biggers [2] has led to the in vitro production of mammalian embryos utilizing synthetic oviductal fluid (SOF) and potassium simplex optimization medium (KSOM). In vitro culture protocols have since been further improved and allowed the development of superior cell-free media [3]. Furthermore, new sequential media have been developed by Gardner and colleagues to address specifically the nutritional needs of the developing embryos at different stages of development [4]. These differences have been designed to meet the changing nutritive requirement of the embryo as a function of their stage of development; therefore culture should take place in different media according to developmental stage [3,4]. However, more research is still needed for a more effective sequential culture system for in vitro produced (IVP) bovine embryos.

Media for IVP embryos not only influences embryo development, but also exerts an effect on embryo survival following cryopreservation. Although IVP bovine embryos have been successful preserved by the use of conventional slow freezing methods [5–8], or standard [5,9] or modified vitrification methods such as open-pulled straws [10], and glass micropipettes (GMP) [11], viability varies among reports and the in vitro conditions under which the IVP bovine embryos are produced prior to cryopreservation seem to affect their ultimate survival [12,13].

Improving in vitro culture condition and modifying cryopreservation technology is an effective approach to increase survival rates of cryopreserved embryos. Embryos cultured in SOF with serum had lower survival rates after vitrification/freezing, but higher cell numbers in hatched blastocysts, than those cultured in the same media with BSA [14,15]. The effect of serum in culture media has been associated with early initiation of blastulation [16–19]. Furthermore, embryos cultured in SOF with no non-essential amino acids have more lipids in their cytoplasm than in vivo embryos [20,21]. The presence of lipids in the cytoplasm, indicated by a dark appearance, is considered detrimental to embryo survival after cryopreservation [22].

Although in vitro production of mammalian embryos has led to the use of several culture media, the combinations of culture media such as KSOM and SOF without co-culture have not been systematically tested previously. The objectives of the present study were to find an improved cell-free in vitro culture system by comparing single versus sequential culture with SOF- or KSOM-based media and to compare post-warming development of IVP bovine embryos following vitrification versus slow freezing.

2. Materials and methods

All chemicals were purchased from Sigma Chemical Co. (St. Louis, MO, USA), unless otherwise indicated.

2.1. Oocyte collection and in vitro maturation

Ovaries were collected at an abattoir (Yankton, SD, USA) and transported to the laboratory within 2–3 h. On arrival, temperature was checked and ovaries were washed with water, then DPBS at 32 °C. Cumulus-oocyte complexes (COCs) from follicles 3–7 mm in diameter were aspirated with a sterile 18-guage needle (Vetpharm, Sioux Center, IA, USA) attached to a disposable syringe at room temperature. Oocytes with intact cumulus cell layers were selected under a stereo-microscope and washed three times in TL-Hepes medium (Bio-Whittaker, Walkersville, MD, USA). Thirty COCs were transferred into a 1.8-ml cryotube (Nunc, Fisher, Pittsburgh, PA, USA) containing M199, 10% FCS (Irradiated FCS, Hyclone, Logan, UT, USA), 1 ml of antibiotic/antimycotic (Gibco BRL, CA, USA) and 10 ng/ml epidermal growth factor (EGF). A 1.8-ml cryotube with IVM media was pre-gassed for 4–5 h in 5% CO_2 in air at 39 °C prior to transferring the COCs into it, and they were shipped to the laboratory in a portable incubator (Minitube, Tiefenbach, Germany) at 38.8 °C by overnight express. On arrival, oocytes were cultured further until 22–23 h of maturation. A total of 2658 COCs were used.

2.2. In vitro fertilization (IVF)

In vitro fertilization in this study was conducted in modified BO media [23]. Briefly, matured oocytes with multiple layers of expanded cumulus cells were washed in TL-Hepes and then in BO fertilization medium supplemented with 6 mg/ml essentially fatty acid-free (FAF)-BSA and 10 μg/ml of heparin. For fertilization, 15–20 COCs were placed in 80 μl of BO medium, under mineral oil, containing frozen-thawed sperm (10^7 sperm/ml) from a single bull for 6 h in 5% CO_2 in air at 39 °C.

2.3. Experiment 1: effect of culture media on embryo development

A total of 1308 presumptive zygotes were removed after 6-h insemination and vortexed to remove cumulus cells. They were then cultured in groups of 25–30 in 25 μl drops of KSOM or SOF under mineral oil in 5% O_2, 5% CO_2 and 90% N_2 in 6-well dishes (EPS, AB Technology, Pullman, WA, USA). Presumptive zygotes were randomly allocated to four culture medium treatments: (1) SOF [16] +5% FCS for 9 days (SOF alone); (2) KSOM + 0.1% FAF–BSA for 4 days, then KSOM + 1% FAF–BSA until Day 9 (KSOM alone); (3) SOF + 5% FCS for 4 days, then KSOM + 1% FAF–BSA until Day 9 (sequential SOF–KSOM); and (4) KSOM + 0.1% FAF–BSA for 4 days, then SOF + 5% FCS until Day 9 (sequential KSOM–SOF). Cleavage and blastocyst rates were recorded 48 h post-insemination (hpi) and following Days 7–9 of culture, respectively.

To determine cell numbers, half of the embryos in each treatment were removed on Day 7 (expanded blastocysts), and the remainder were left to continue their development until Day 8 (hatched blastocysts). Expanded and hatched blastocysts were mounted on a slide, stained with Hoechst 33,342 (10 μg/ml with 20% glycerol in DPBS), and covered with a cover slip. The total number of cells in blastocyts was determined by counting the number of nuclei under epifluorescent microscopy.

2.4. Experiment 2: post-warming survival of IVF embryos cultured in different media

The post-cryopreservation viability of embryos produced from the best medium, KSOM–SOF as determined in Experiment 1, was tested with two cryopreservation methods: vitrification and conventional slow freezing. The vitrification protocol used in this experiment was adopted from Rall [5] and Dinnyés et al. [24] with minor modification. Briefly, 0.25-ml plastic straws were prepared prior to vitrification by pushing a 2-cm cotton plug into the straw, and placing a 7.5-cm column of 1.0 M sucrose in DPBS into the straw using a 1-ml syringe with a 27-gauge needle (Vetpharm). Then, a 1-cm column of vitrification solution (VS3a) was placed adjacent to the sucrose column but separated by a 0.5 cm air space. The straw was heat-sealed at the plug end and placed at $-20\,^{\circ}$C prior to vitrification. Placing the straw at $-20\,^{\circ}$C was a modification of the original VS3a protocol to reduce the toxicity associated with high concentrations of cryoprotectants at ambient temperature [25], because of the time required to load the embryos into straws and to seal the straws before plunging them into liquid nitrogen [26,27]. Furthermore, a sealed 0.25-ml straw, filled with solutions and air, is susceptible to extreme pressure when directly plunged into liquid nitrogen without first being cooled [28]. The notion of pre-cooling the straw was also because embryos were exposed to high concentrations of cryoprotectant at room temperatures during the time of loading and sealing straw, although this factor, which may influence survival, has not been extensively studied [25–27].

For vitrification, Day-7 blastocysts were equilibrated in VS3a (6.5 M glycerol in DPBS with 6% w/v BSA) in three steps at room temperature. Firstly, embryos were placed for 3 min in DPBS with 6% BSA (PB1) and then equilibrated in 25% of VS3a (1.6 M glycerol with 6% BSA in DPBS) for 20 min. Embryos were then rinsed for 30 s in 65% VS3a (4.2 M glycerol and 6% BSA in DPBS) and transferred into a column of 100% VS3a in a 0.25-ml straw which was from $-20\,^{\circ}$C as described above. The straw was heat sealed and left at room temperature for 1 min, then held in liquid nitrogen (LN_2) vapor (-150 to $-180\,^{\circ}$C) for 3 min and then placed directly into LN_2. Straws were stored for 1 week to several months in LN_2 ($-196\,^{\circ}$C).

Warming was accomplished by holding the frozen straw for 10 s in air; 10 s in a 22 $^{\circ}$C water bath, then shaking vigorously to mix the contents within the straw. The sealed end of the straw was cut and its contents emptied into a Petri dish and embryos were recovered and transferred into 1.0 M sucrose and then 0.5 M sucrose for 2 min each. Finally, embryos were re-hydrated in mPBS (3 mg/ml BSA in DPBS) for 4–5 min, washed and transferred into culture media (SOF + 7.5% FCS). Re-expansion and development of embryos and hatching of blastocysts were recorded at 6, 24 and 48 h post-thawing.

For a conventional embryo slow freezing, Trans Ova's standard procedure was used, with a modification of replacing ethylene glycol with glycerol [29], in order to use same cryoprotectant as in vitrification. Briefly, a column (6 cm) of 0.5 M sucrose in mPBS was placed into a 0.25-ml plastic straw using a 1-ml syringe with a 27-gauge needle. It was followed by a 0.5 cm air space after which 0.5 cm of freezing solution (10% glycerol in DPBS with 6% BSA) was loaded. This was followed by another 0.5 cm air space and then loading of another 1 cm of freezing solution as described by Schiewe et al. [30]. The plug end of the straw was heat sealed, prior to the embryos being placed inside the freezing solution, and maintained at 24 $^{\circ}$C until the equilibrated embryos were loaded.

To freeze the embryos, Day-7 blastocysts were placed in PB1 for 3 min and equilibrated for 5 min in 0.68 M glycerol in PB1. Embryos were transferred into a column of 1.36 M glycerol solution in a 0.25-ml straw at room temperature (24 °C) and left for 10–15 min. The straws were then placed into the alcohol bath of a programmable freezer (Bio Cool IV, Stone Ridge, NY, USA) at −5.5 °C and seeded by touching the wall of the straw with cold forceps after 10 min. Ice crystal normally formed within 10–15 min. The straws were then cooled from −5.5 to −32 °C at a rate of 0.6 °C/min. After reaching the target final temperature of −32 °C, straws were transferred into LN_2 and stored for 1 week to several months.

Warming of a frozen straw was accomplished by holding it for 10 s in air; 10 s in a 22 °C water bath. The straw was then shaken vigorously to mix the contents and emptied into a Petri dish. Recovered embryos were transferred into 0.25 M sucrose for 2 min and re-hydrated in mPBS (3 mg/ml BSA in DPBS) for 5 min. Embryos were recovered and then washed, and cultured in SOF + 7.5% FCS. Re-expanded, further developed or hatched embryos were evaluated at 6, 24 and 48 h post-thawing.

2.5. Embryo transfer

The viability of embryos ($n = 10$) produced by the sequential KSOM–SOF culture system was tested following vitrification/warming (VS3a) by direct non-surgical transfer into 10 synchronized recipients. Pregnancy was detected by ultrasound on Day 75 following transfer.

2.6. Statistical analysis

Data were analyzed using the GENMOD procedure of SAS; this procedure transforms the data into logits and then fits a generalized linear model to the data (SAS; Cary, NC, USA). Experiment 1 was analyzed using the GLM procedure of SAS. The Chi square test was used for Experiment 2. Cell numbers were assessed by ANOVA. All values given are significant at $P < 0.05$.

3. Results

3.1. Experiment 1

The IVF embryos were cultured in four different culture media and their in vitro developments were compared. As shown in Table 1, there were no significant differences in any of the developmental parameters monitored were seen between embryos cultured in SOF or KSOM alone or SOF followed by KSOM (Treatments 1, 2 and 3). However, the culture system of KSOM + BSA for the entire duration of the culturing period (Treatment 2) yielded lower ($P < 0.05$) rates of development on Day 6 of culture. However, this effect was eliminated when sequential KSOM culture followed by SOF treatment was used (Treatment 4). The reverse sequential treatment by SOF followed by KSOM (Treatment 3) did not have any effect on embryo development, which was not different from that

Table 1
Effect of KSOM and SOF on in vitro development of bovine embryos

Treatment	Number of oocytes	Morulae (%) (Day 5)	Early blastocysts (%) (Day 6)	Blastocysts (%) (Day 7)	Total blastocysts (%) (Days 7–9)
SOF	327	40 ± 3.0^{ab}	22 ± 1.8^{ab}	31 ± 3.4^{ab}	38 ± 3.4^{a}
KSOM	327	38 ± 3.0^{b}	7 ± 1.8^{c}	28 ± 3.4^{b}	43 ± 3.4^{a}
SOF–KSOM	327	43 ± 3.0^{ab}	20 ± 1.8^{b}	34 ± 3.4^{ab}	40 ± 3.4^{a}
KSOM–SOF	327	47 ± 3.0^{a}	26 ± 1.8^{a}	36 ± 3.4^{a}	45 ± 3.4^{a}

$^{a-c}$ Within columns, values with different superscript are different ($P < 0.05$).

of embryos cultured in SOF or KSOM alone throughout the culture period (Treatments 1 and 2).

Total hatching rate was also higher ($P < 0.05$) when embryos were cultured in sequential KSOM–SOF culture system compared to other treatments (Fig. 1). The hatching rate of embryos cultured in KSOM alone (Treatment 2) was significantly lower ($P < 0.05$) on Days 7 and 8, when compared to other treatments (Fig. 2).

Despite the slow development, embryos cultured in KSOM alone (Treatment 2) had well defined cluster of cells within blastocysts (morphological assessment), compared to those cultured in SOF or SOF–KSOM. When total cell numbers were determined (Table 2), sequential culture with KSOM–BSA for 4 days followed by SOF + FCS culture (Treatment 4) increased ($P < 0.05$) the total cell numbers when compared to those embryos cultured in KSOM + BSA alone (Treatment 2). In contrast, embryos cultured in reverse
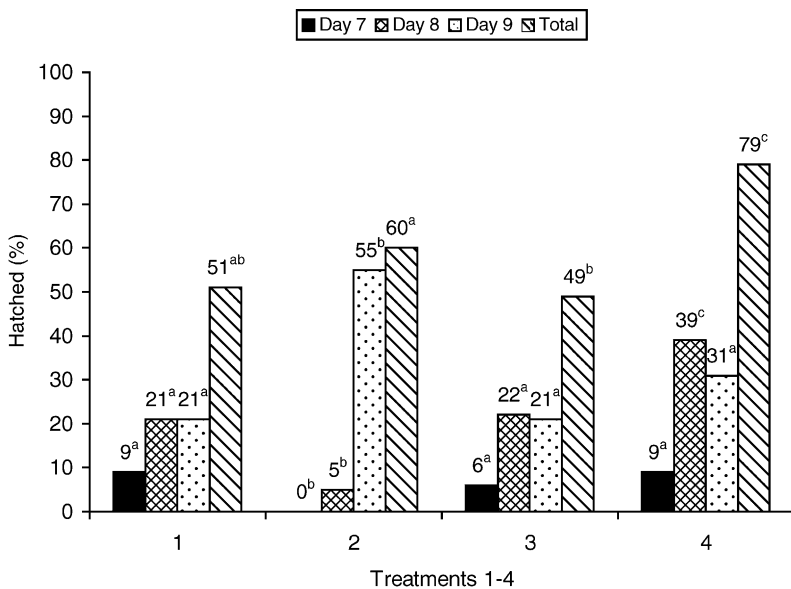
$^{a-c}$Within columns, values with different superscripts are different (P < 0.05)

Fig. 1. Effect of SOF/KSOM on hatching rate of IVP bovine blastocysts.

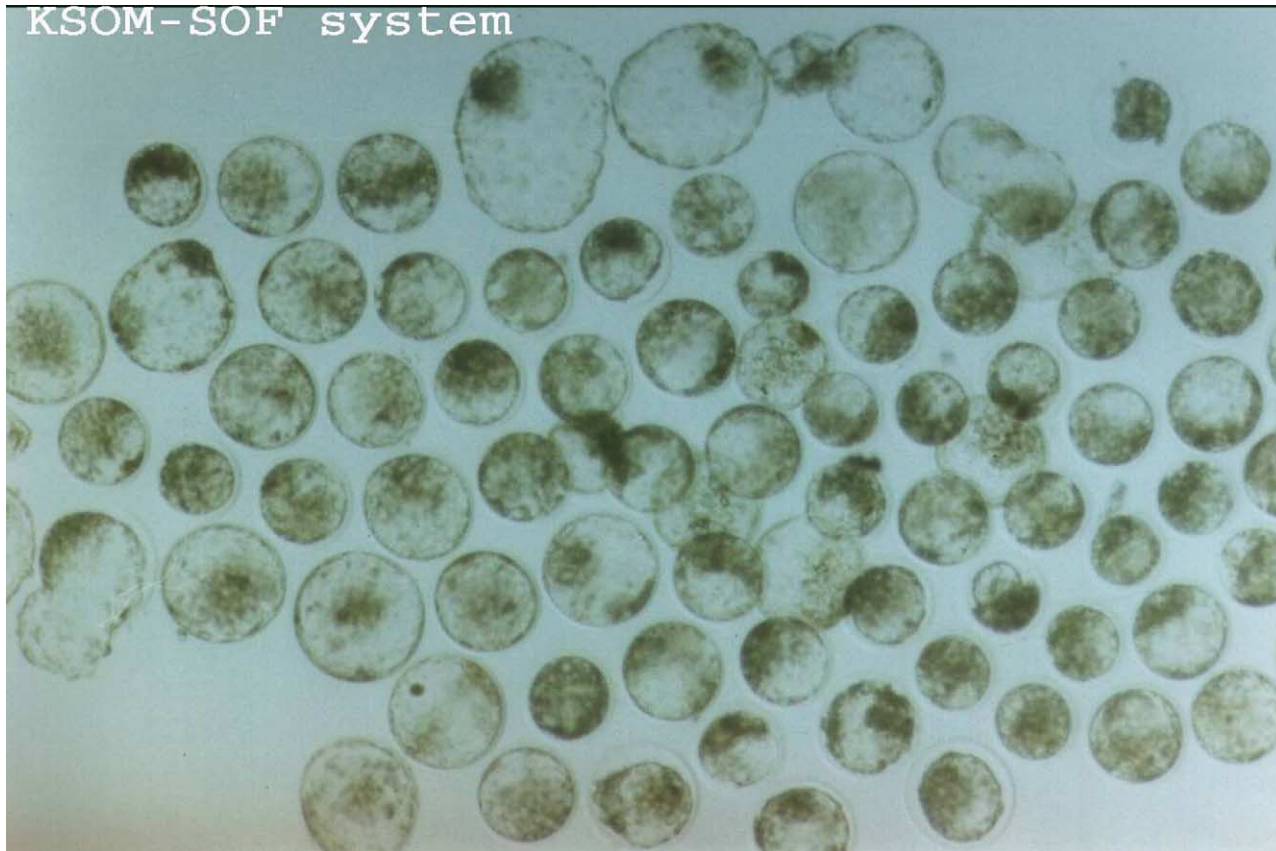Fig. 2. In vitro produced bovine embryos from KSOM–SOF culture system on Day 7 (magnification 100×).

Table 2
The total average number of cells of in vitro produced bovine embryos

Treatment	Number of embryos	Mean (±S.E.M.) total cell numbers of embryos	
		Expanded (Day 7)	Hatched (Day 8)
SOF	8	115 ± 4.3[ab]	187 ± 9.7[a]
KSOM	8	99 ± 4.3[bc]	175 ± 9.7[a]
SOF–KSOM	8	84 ± 4.3[c]	146 ± 9.7[a]
KSOM–SOF	8	127 ± 4.3[a]	268 ± 9.7[b]

[a–c] Within columns, values with different superscript are different ($P < 0.05$).

sequential with SOF first followed by KSOM (Treatment 3) resulted in lower ($P < 0.05$) total cell numbers on Day 7 (expanded blastocysts) and their inner cell mass was not as clearly clustered as in blastocysts grown in the other three culture systems (Fig. 3). Overall, embryos cultured in sequential culture of KSOM–SOF (Treatment 4) had the best Day-7 development, higher ($P < 0.05$) hatching rate and most cell numbers. Therefore, this culture system was selected for further cryopreservation testing.

3.2. Experiment 2

The best culture system (sequential KSOM–SOF culture) from Experiment 1 was selected and used for embryo cryopreservation viability testing by either vitrification or conventional slow freezing. Data are presented in Table 3. Post-warming development and hatching rates of embryos vitrified in VS3a were consistent ($P < 0.05$) at all evaluation times (i.e. 6, 24 and 48 h post-warning) than embryos frozen by the slow freezing method. Morphological observations showed that more cells had fragmentation or damage when
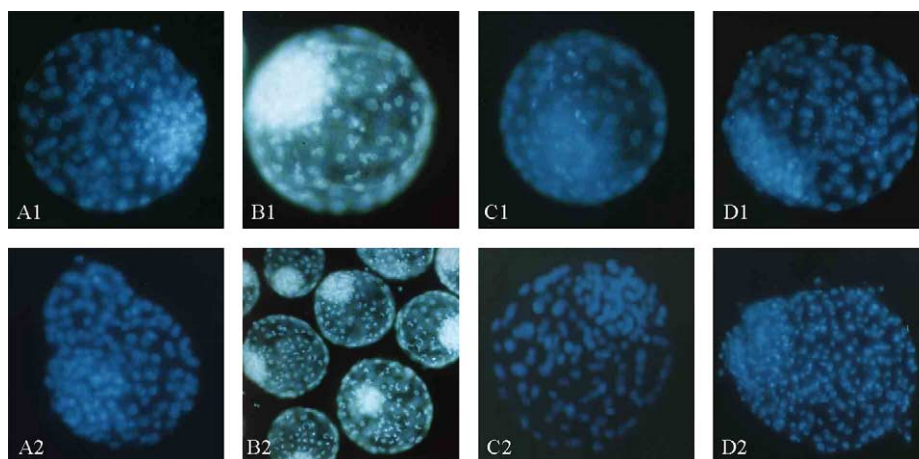

Fig. 3. IVP bovine expanded/hatched blastocysts stained with Hoechst 33,342 on Day 7: A1 (SOF alone); B1 (KSOM alone); C1 (SOF–KSOM); D1 (KSOM–SOF) and Day 8: A2 (SOF alone); B2 (KSOM alone); C2 (SOF–KSOM); D2 (KSOM–SOF) (magnification 200×, except for B2, which is at 100× magnification).

Table 3
The number of blastocysts re-expanding and developing post-vitrification vs. slow freezing

Treatment	Number of embryos	Post-warming development			
		6 h	24 h	48 h	Hatched
VS3a	297	212 (71%)[a]	190 (64%)[a]	179 (60%)[a]	160 (54%)[a]
Slow freezing	297	143 (48%)[b]	118 (40%)[b]	91 (31%)[b]	66 (22%)[b]

[ab] Within columns, values with different superscript are different ($P < 0.05$).

embryos were slow frozen/warmed, than when embryos were vitrified/warmed by VS3a. Vitrified embryos re-expanded earlier and more vitrified embryos developed further than embryos frozen by the slow freezing method (Table 3).

To determine the in vivo developmental potential of embryos subjected to the sequential KSOM–SOF culture and the vitrification protocols from Experiment 2, a total of 10 vitrified IVP embryos were directly transferred to synchronized recipients and resulted in an ultrasound confirmed 30% pregnancy rate in 75 days of gestation.

4. Discussion

To date, much effort has been focused on substituting serum/BSA with chemically defined macromolecules such as polyvinyl alcohol (PVA) and sodium hyaluronate (SH) in vitro culture systems [31,32] and for freezing [8,33–35]. However, results obtained with these substitutes have been either inconsistent or inferior to those obtained with serum/BSA ([31,32], review [36]). Therefore, a culture and freezing system utilizing serum/BSA remains the best choice for in vitro embryo production. In the present study, sequential KSOM + BSA culture followed by SOF + FCS (Treatment 4) resulted in improved morulae, and early and Day-7 blastocyst rates compared to KSOM + BSA alone (Treatment 2). In contract, the reverse sequential treatment by SOF then KSOM (Treatment 3) did not improve development in any criteria tested. Additionally, the sequential KSOM–SOF system produced blastocysts with highest ($P < 0.05$) cell numbers and morphologically showed best quality blastocysts based on the IETS standard.

The superiority of the KSOM–SOF system might have resulted from the sequential use of BSA and serum because, in the present study, the use of BSA or serum alone or the use of serum first and then BSA produced embryos with lower hatching rates and Day-7 expanded blastocysts with lower cell numbers. These observations may be related to a beneficial effect of serum during the later stages of embryo development [3,4]. The lack of serum during the later stages of culture (KSOM + BSA alone or Treatment 2) resulted in lower blastocyst rates and fewer cell numbers on Day 7, suggesting the importance of serum during this period. The beneficial effect of serum in embryo culture has been reported previously, and it has been shown that serum stimulated early expansion of blastocysts by accelerating the blastulation process [17,18,37–40] and improve blastocyst yield [17]. This was consistent with our observation that the number of Day-6 embryos resulting from culture in KSOM + BSA alone (Treatment 2) was significantly lower than those in other groups, suggesting that without the addition of serum, there is delayed blastulation.

Furthermore, serum in culture media has been reported to promote hatching by providing a pool of plasminogen; bovine embryos convert it to plasmin that proteolytically degrades the zona pellucida and facilitates hatching [41]. Van Soom et al. [42] reported that hatching of in vivo embryos was predominantly on Day 9. Therefore, the presence of only BSA in the KSOM alone might have prolonged compaction of morulae and delayed blastulation. It has been speculated that the prolonged state of compaction allows for a better allocation of ICM cells in vivo embryos [42]. This may also hold true for in vitro produced embryos, since in our study embryos cultured in KSOM + BSA delayed blastulation.

Despite similar overall blastocyst rates among the three treatments, the advantage of using BSA and serum sequentially was that embryos cultured in the presence of serum throughout their culture period (SOF + FCS alone; Treatment 1), or in media that lacked serum during the second half of the culture period (SOF + FCS then KSOM + BSA; Treatment 3) had much reduced cell numbers compared to those cultured with a sequential use of BSA and serum (KSOM + BSA then SOF + FCS; Treatment 4). These results were consistent with reports by Yoshioka et al. [15], Tricoire et al. [40] and Kuran et al. [43] that used serum in SOF media during the entire embryo culture and found that serum induced early blastocyst formation without increasing cell proliferation. Collectively, it appears that serum is beneficial only when used during later stages of embryo development, and furthermore, that inclusion of serum during the early stages will reduce embryo quality.

In addition to serum, the superior embryo development observed when the sequential use of KSOM + BSA and SOF + FCS was used may be related to the contribution of citric acid in the SOF medium used during that later stage of embryo culture. It has been reported that citric acid improves embryo development during the later stages of bovine and rabbit embryo culture [16,44–46]. In addition to producing more embryos and embryos with greater cell numbers, culture in KSOM + BSA and then SOF + FCS (Treatment 4) also yielded embryos with a better morphology (on Day 7) than the other treatments. Furthermore, they were lighter in color and tightly compacted [22], consistent with the previous report by Holm et al. [16]. The color of the cytoplasm in IVMFC embryos has been associated to the lipid to protein ratio, a factor influencing sensitivity to chilling and cryopreservation of bovine [34,47] embryos. The lighter color of embryos generated in the KSOM–SOF system suggested that they could have improved survival following cryo-preservation.

In Experiment 2, controlled slow freezing was compared to vitrification (VS3a) of embryos produced from the most effective culture system from Experiment 1, i.e. KSOM + BSA and then SOF + FCS (sequential KSOM–SOF; Treatment 4). More of the embryos that had been vitrified/warmed in VS3a re-expanded, developed, and hatched than those frozen/warmed by a slow freezing method. These results confirmed the previous report by Mahmoudzadeh et al. [48] who recorded lower survival of IVP embryos following slow freezing than vitrification and attributed this to a higher sensitivity of the IVP embryos to slow cooling [8,49]. Enright et al. [14] also reported that in vitro produced bovine embryos cultured in SOF with 10% FCS did not survive slow freezing, and that VS3a resulted in an improved, albeit still low, survival rate. Slow warming of blastocysts has also been associated with lower survival at all cooling rates [49]. A recent report by Crosier et al. [50] indicated that embryos produced either in TCM 199 or SOF media with non-essential amino acids contained more lipids in their cytoplasm than in vivo

embryos, which make them more sensitive to damage through cryopreservation. Therefore, given the additional evidence of our study, vitrification was less injurious to bovine embryos produced in sequential KSOM–SOF than slow freezing. However, embryos produced from a co-culture system and frozen/thawed by the same slow freezing method used in this study had a survival rate ranging from 80 to 100% (Nedambale and Groen, unpublished data). Furthermore, the slow freezing method used in this study might have been sub-optimal for embryos produced from sequential KSOM–SOF culture system.

When embryos produced from the KSOM–SOF system were tested in utero (single direct transfer without post-warming selection), a 30% pregnancy rate resulted. Due to the limited availability and high cost of recipient females, only a small number ($n = 10$) of embryos were transferred in the present study. Our pregnancy rate was consistent with the 24% pregnancy rate recorded by Dinnyés et al. [24] and comparable to those of Massip et al. [51] who recorded a pregnancy rate of 33% following transfer of vitrified embryos after post-warming selection, and seemed considerably better than the 14% pregnancy rate achieved following transfer of slow frozen/warmed embryos [5]. A large field trial to test pregnancy rates for embryos produced from sequential KSOM–SOF and cryopreserved by VS3a is needed to further test and confirm the viability of these embryos.

In conclusion, replacing KSOM + 0.1% BSA with SOF + 5% FCS after 4 days of embryo culture increased early embryo development and promoted a higher hatching rate. Additionally, embryos generated from the KSOM–SOF system appeared to produce better quality blastocysts with greatest total cell numbers. Vitrification using VS3a may be used more effectively to cropreserve in vitro produced embryos from KSOM–SOF than the conventional slow freezing method.

Acknowledgements

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